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EMBRYOGENY OF *PHASEOLUS*: DEVELOPMENTAL PATTERN OF LACTATE AND ALCOHOL DEHYDROGENASES

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Key Word Index—*Phaseolus*; Leguminosae; bean; lactate dehydrogenase; alcohol dehydrogenase; embryo development.

Abstract—Alcohol and lactate dehydrogenase activity and their electrophoretic isoenzymes were determined in developing *Phaseolus vulgaris* embryos, seed coats and pods. Alcohol dehydrogenase activity decreased from the embryo out to the pod whereas lactate dehydrogenase activity increased. The two activities showed opposite profiles with respect to time, from day 10 to 18 in the embryo. Alcohol dehydrogenase isoenzyme pattern differed in different organs whereas lactate dehydrogenase was always present as five isoenzymes.

INTRODUCTION

The developing angiosperm embryo is surrounded by tissues from which it derives its nutrients, i.e. the seed coat and the fruit wall. These tissues could constitute a substantial barrier to oxygen diffusion to the embryo. Oxygen tension within the developing seeds of *Haemanthus* and *Clivia* was found to be very low [1], suggesting that the embryonic environment is, indeed, anaerobic.

As part of a continuing investigation of bean embryogeny, we studied two dehydrogenases involved in anaerobic metabolism—lactate dehydrogenase (LDH) and alcohol dehydrogenase (ADH). Both of these enzymes are present in plants, especially those under an oxygen deficit. In some plants, anaerobic stress induces the expression of otherwise non-expressed ADH genes [2–4] and causes an increase in ethanol production in many plants [5]. LDH activity is present in many plants under anaerobic stress; in particular, storage tissue [6–10] and germinating or imbibing seeds [11, 12]. It has also been isolated from lettuce leaves in one of the few reports of LDH activity in photosynthetically active higher plant tissue [13]. LDH activity in naturally developing seeds has not yet been investigated.

Multiple forms (isoenzymes) of these dehydrogenases are present in both plants and animals [9, 10, 14, 15]. Isoenzymes often have different metabolic roles, the requirement for which might change during development. Therefore, a qualitative study of the isoenzymes was undertaken to determine if organ specific patterns exist and whether these patterns change during the development of bean fruits. The results of these studies will serve as background for the function of the dehydrogenases during embryo development and will provide insight into the environment of the developing bean embryo.

RESULTS AND DISCUSSION

Change in ADH activity expressed both as specific activity and on a whole organ basis are shown in Figs. 1 and 2, respectively. ADH activity in the embryo and whole seed increased steadily to reach its highest level of 0.355 units/mg protein in the day 18 embryo. Subsequently, specific activity decreased because of the build-up of metabolically inert storage proteins(Fig. 1). However, on a per organ basis, the actual activity in the whole embryo remained high through maturation (Fig. 2). ADH activity within the separated cotyledons and axis showed similar changes to those of the whole embryo (data not shown).

LDH, on the other hand, had a lower activity in the embryo throughout development (Figs. 3 and 4). Unlike

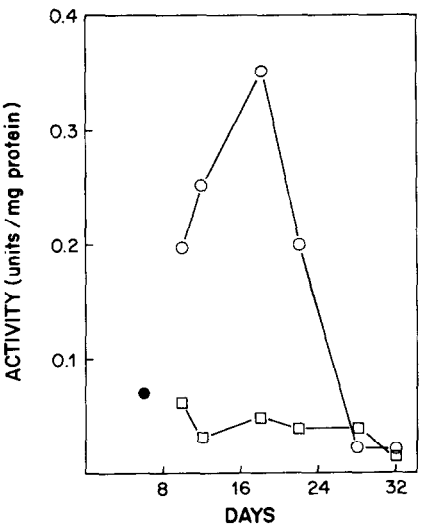

Fig. 1. ADH activities within developing bean fruits. Enzyme activity is expressed as units per mg protein. Time represents days after fertilization. (●) Whole seed; (○) embryo; (□) seed coat.

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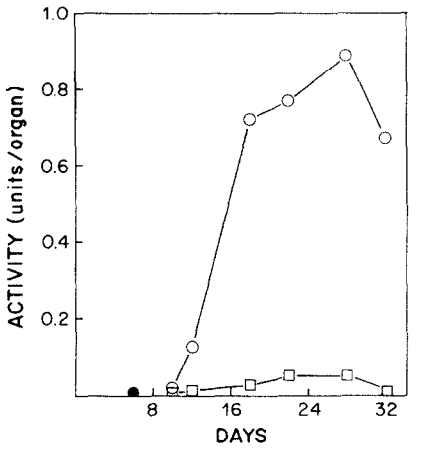

Fig. 2. ADH activities within developing bean fruits. Enzyme activity is expressed as units per whole organ. Time represents days after fertilization. (●) Whole seed; (○) embryo; (□) seed coat.

ADH, its specific activity was highest during early embryo development followed by a continuous and gradual decline (Fig. 3). Thus, pyruvate reduction to lactate seems to be the major fermentation pathway in the very young developing embryo, and assumes a less dominant role relative to ADH in the older embryo. Other investigations show that lactate production precedes ethanol production [16]. This suggests that LDH is the more active of the two dehydrogenases during the initial stages of anaerobiosis. The large production of lactate and consequent acidity is thought to inhibit LDH (which has a neutral pH optimum and is inhibited by ATP at low pH) but to stimulate

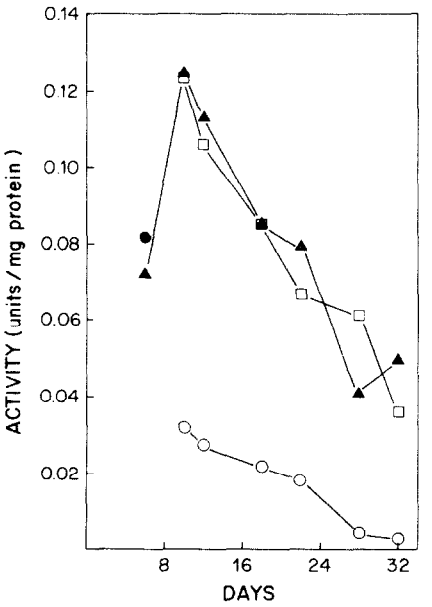

Fig. 3. LDH activities within developing bean fruits. Enzyme activity is expressed as units per mg protein. Time represents days after fertilization. (●) Whole seed; (○) embryo; (□) seed coat; (▲) pod.

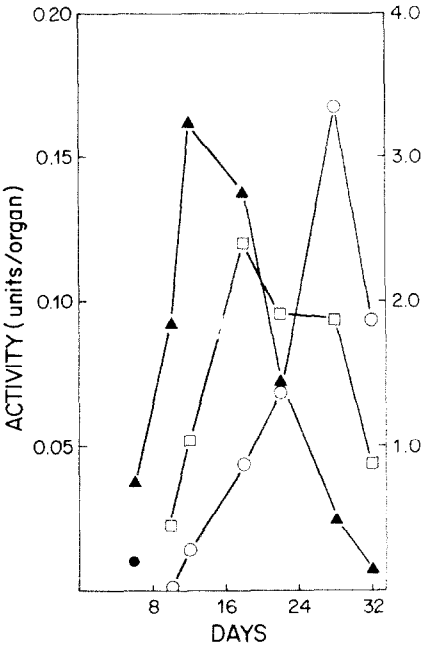

Fig. 4. LDH activities within developing bean fruits. Enzyme activity is expressed as units per whole organ. Left-hand scale represents the activities of the whole seed (●) and embryo (○), and the right-hand scale represents the activities of the seed coat (□) and pod (▲).

pyruvate carboxylase and ADH (which have lower pH optima) [16, 17]. Our observation of increasing ADH activity and decreasing LDH activity supports this hypothesis.

Another observation of note is the increasing activity of LDH relative to ADH from the embryo out to the pod. ADH activity was greater than LDH activity in the embryo, less than LDH activity in the seed coat and undetectable in the pod (where LDH activity was quite high) (Figs. 1–4). Thus ADH seems to be playing a decreasing proportional role in fermentation as the tissue becomes less anaerobic. In both the pod and the seed coat, LDH activity declined gradually from day 10. ADH activity in the seed coat remained low throughout development and showed no significant changes.

ADH has organ specific isoenzymes (Fig. 5). Three isoenzymes are present in the whole embryo and the embryonic axis (Fig. 5a). During the maturation stage, an additional fast-migrating isoenzyme appeared in the axis (Fig. 5b). In the cotyledon, a strongly stained slow-migrating isoenzyme was always present in addition to the fast-migrating isoenzymes, as shown in Figs. 5c and 5d. These organ specific isoenzymes may have different structural and functional roles at different stages of development if their substrate specificities, inhibition properties and other kinetic characteristics do vary *in vitro* as do those of other isoenzymes [14].

Unlike ADH, LDH was present in five electrophoretically distinct forms in all the organs. The relative mobilities of the five isoenzymes were 0.27, 0.29, 0.31, 0.33 and 0.36. This LDH resembles the five LDH isoenzymes of potato [9, 10]. A recent study by Jervis [10] provides evidence that the potato LDH, like that of vertebrates, is

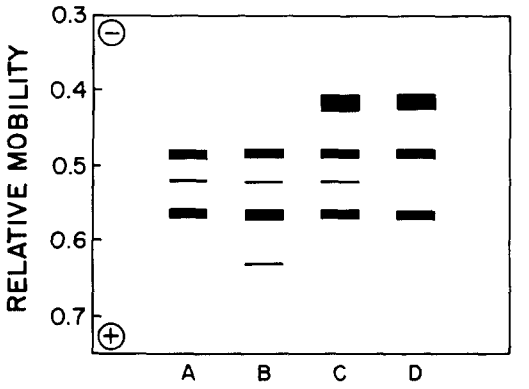

Fig. 5. Polyacrylamide gel electrophoresis patterns of ADH. Column A represents the isoenzyme patterns from extracts of whole embryos (up to day 12 after fertilization) as well as developing embryonic axes (up to day 22). Column B represents the isoenzyme pattern from 28- to 32-day-old embryonic axes. Column C represents the isoenzyme pattern from 18-day-old cotyledons and column D represents the isoenzyme pattern of 22- to 32-day-old cotyledons.

an association of two different subunits. All of the five isoenzymes of potato have not been completely separated and a low specific activity of LDH in most starting material hinders purification. Since the bean pods have a comparatively high specific activity of LDH and contain all five isoenzymes, they should prove to be a good source for enzyme purification and characterization of individual isoenzymes.

The presence of both dehydrogenases in the bean fruit and studies of the oxygen tension surrounding the embryo [1] indicate that the embryonic environment is anaerobic. The anaerobic environment may play an important role in embryo development. A recent experimental study on pollen embryo induction indicated that anaerobiosis has a stimulatory effect on both the number of plantlets formed per anther and on the rate of growth of the plantlets [18]. Furthermore, a lowering of dissolved oxygen in cell suspension cultures of carrot tissue also increased the number of somatic embryos formed in culture [19]. Thus, anaerobiosis may be required to stimulate plant cells to express their embryonic potential.

EXPERIMENTAL

Developing seeds were obtained from greenhouse-grown *Phaseolus vulgaris* var. Taylor's Horticultural. The general post-fertilization develoment of *P. vulgaris* has been described in ref. [20]. The successive developmental stages of the embryo can be identified by the pod and seed lengths. These measurements were therefore used to stage the embryos.

Different parts of the seed were dissected, separated into component parts, weighed and either extracted immediately or frozen in liquid N_2 and stored at $-20°$ until use.

Enzyme extraction. Samples were homogenized in an ice-cold 0.05 M Tris–HCl buffer (pH 7.2) containing 2% (v/v) Triton X-100, 1% (w/v) polyvinylpolypyrrolidone and 4 mM dithiothreitol. The extracts were centrifuged at 26 000 *g* for 30 min in a Sorvall RC-5B refrigerated centrifuge at 0–4°. The supernatants were kept on ice and assayed at once.

Enzyme assays. Alcohol dehydrogenase activity was determined by a modification of the method described in ref [21]. The assay mixture for ADH contained pyrophosphate–glycine buffer (78 mM in pyrophosphate, pH 8.7), semicarbazide–HCl (75 mM), reduced glutathione (1 mM), EtOH (150 mM), NAD (1 mM), and extract (0.1 ml) in a total vol. of 1.5 ml. LDH activity was measured in the direction of pyruvate reduction. The assay mixture contained Tris–HCl buffer (5 mM, pH 7), Na pyruvate (10 mM), NADH (0.1 mM) and 0.2 ml of extract in a total vol. of 1.5 ml. For both activities, the change in *A* at 340 nm was monitored with a spectrophotometer and recorder. The assaying conditions, i.e. pHs, substrate and cofactor concentrations, were experimentally determined to be the optimum for these enzymes. One unit of activity is equal to 1 μmol pyridine nucleotide reduced or oxidized per min.

Total protein was measured by the method of ref. [22]. Bovine serum albumin was used as standard. Results are the means of at least three replicates.

Acrylamide gel electrophoresis and isoenzyme staining. The crude extracts were separated on a micro-thin layer electrophoretic system as described in ref. [23]. The conditions used were essentially the same, except that a 0.3 M Tris–citrate buffer (pH 8.7) was used in the running gel and a 0.15 M Tris–citrate buffer (pH 6.8) was used in the stacking gel. Running gel concentration was 7%.

The isoenzymes were localized in gels using the following incubation media. For alcohol dehydrogenase, gels were stained with a soln containing 50 mM Tris–HCl (pH 8), 340 mM EtOH, 0.75 mM NAD, 65 mM phenazine methosulfate, 0.4 mM nitroblue tetrazolium and 0.02 mM KCN. For lactic dehydrogenase, gels were stained with a soln containing 42.5 mM Tris–HCl (pH 7.1), 10 mM Na lactate, 0.75 mM NAD, 65 mM phenazine methosulfate, 0.4 mM nitroblue tetrazolium and 0.02 mM KCN.

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